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Training and Research Accomplishments

This year I have completed preliminary work on the project, "Pim-1, a potential participant in breast cancer". All work done to date has concentrated on those experiments outlined in Task 1 of the "Statement of Work". Initial studies have been completed showing by Western blot that Pim-1 protein expression can be induced by prolactin, as well as with dexamethasone and insulin which are often used together with prolactin when looking at genes regulated by this lactogen. This work needs to be completed with repeated experiments using the proper controls. I have recently contacted Dr. Wolfgang Doppler (University of Innsbruck, Austria) for the human β -casein promoter-CAT construct and he has agreed to send this to me for use as a control in these and other experiments. Since I am looking at a system where there are site-specific interactions between proteins and DNA and there are slight variations in protein and DNA sequence between species, I feel it is important to use all human components, such as human Stat5a constructs and human β -casein constructs. However Dr. Doppler has also recommended that I use the rat β -casein promoter reporter construct and I have requested this as well.

I have recently established a Northern blot protocol using a non-radioactive detection system where I have been able to show that two different human mammary epithelial cell lines, MCF-7 and T47D-co, contain different basal levels of *pim-1* message. With this protocol, I will be able to determine the level of *pim-1* message as well as protein when the cells are stimulated with prolactin and when the cells are transfected with the Stat5a constructs. Determining *pim-1*

message levels when the cells are stimulated with prolactin and/or transfected with the Stat5a constructs is not written as one of the experiments in Task 1 of the "Statement of Work" but I feel on re-evaluation of the tasks, that it is an important aspect. This is because Stat5a is a transcription factor and Pim-1 expression has been shown to be regulated at many different levels, including translation. Therefore increases in *pim-1* message expression may not be observed to the full extent if only Pim-1 protein levels are examined because there may be regulation of translation occurring.

I have been able to successfully generate an expression vector containing the wild type human Stat5a (pBK-CMV(Δ lac)(wtStat5a)) as well as an expression vector containing the constitutively active form of human Stat5a (pBK-CMV(Δ lac)(Stat5aN642H)). I have carried out an extensive search of the literature and have not found a published report using the human form of a constitutively active or dominant negative Stat5a. Therefore, I have worked on making mutations which have already been published for the mouse Stat5a mutants. This constitutively active mutant was modeled after that generated by Ariyoshi *et al.* (JBC 275:24407, 2000) where they characterized a constitutively active form of mouse Stat5a. I am still in the process of determining whether the specific point mutation introduced in the human sequence results in the same constitutive activation effects seen with the mouse protein. Both the wild type and constitutively active constructs have been sequenced and since the Stat5a protein has a Flag-tag attached to it, their expression in MCF-7 cells has been verified by Western blot using antibody to the Flag-tag. Unexpected difficulties have been encountered in the construction of the expression vector containing the dominant negative form of Stat5a (protein truncated at amino acid 750 modeled after the construct generated by Moriggl *et al.* (Mol Cell Biol 16:5691, 1996) with the mouse Stat5a). DNA sequencing results of a number of potential clones have shown that there were multiple mutations in the sequence of each clone, with no two clones containing mutations at the same site. At this time, I do not know the cause of these mutations. The elongation temperature at which the PCR is running is the optimal temperature for the polymerase being used and therefore should not theoretically be introducing mutations into the

sequence. Therefore further subcloning is being performed to obtain the dominant negative form of Stat5a.

At this point in my work I am concentrating on examining the effects of wild type Stat5a, the constitutively active Stat5a mutant, and the dominant negative Stat5a mutant to determine whether this transcription factor is capable of regulating the expression of *pim-1* in human mammary epithelial cells. At the present time, the construction of the Jak2 dominant negative mutant is on hold since the important interaction of the pathway involves Stat5a and its direct regulation of *pim-1* expression. If these results are satisfactory in relating the regulation of *pim-1* by prolactin through the Jak/Stat pathway, it may be deemed unnecessary to go through with this part of the Task and rather continue on with the rest of the proposed research which may answer more important questions. I am currently working with the wild type and constitutively active form of Stat5a constructs in transfections of MCF-7 cells to see whether the overexpression of these proteins results in an alteration of *pim-1* levels. Preliminary results for Pim-1 protein expression of cells transfected with the constitutively active form of Stat5a shows little change in Pim-1 protein levels. These results from Western blots of MCF-7 cells transfected with constitutively active Stat5a suggest that there may be translational regulation occurring which prevents the production of Pim-1 protein thus masking the increase in *pim-1* message level that may be occurring. Therefore, these transfections will be repeated and Northern blots performed to examine message levels of *pim-1*. Another possibility exists as to why there is no increase seen in Pim-1 protein levels in cells transfected with the constitutively active Stat5a. This possibility is that additional signals resulting from cells grown on extracellular matrix may be required to obtain optimal induction of *pim-1*. There are many components of the extracellular matrix that have been determined to be important in the activation of Stat5a and the induction of β -casein expression, a gene shown to be regulated by Stat5a (PNAS 87:9118, 1990). These signals may be needed for the induction of *pim-1* message by Stat5a in mammary epithelial cells. Since I have not come across any literature which has examined the expression of a Stat5a-mediated transcriptional induction of a gene using human mammary epithelial cells, I will

perform experiments modeled after those done with mouse mammary epithelial cells but using human mammary epithelial cells. It appears at the moment that using a collagen gel may be an appropriate method to induce the expression of *pim-1* message when the cells are transfected with the constitutively active form of Stat5a. This has shown to be successful in the induction of β -casein by prolactin for mouse mammary epithelial cells. Therefore, this aspect of the research will be examined and determined whether necessary to induce the expression of *pim-1* message along with activation of Stat5a.

Addition to the project

I would also like to take a survey of human mammary tissue, both normal and cancerous, to see what proportion of cancer cases express increased Pim-1 protein in epithelial cells. Preliminary results carried out by our lab has shown increased Pim-1 protein expression in the human mammary carcinomas and initial studies that I have completed this past year agree with this finding. I feel that this data is very important as background and further evidence that Pim-1 is involved in the induction of breast carcinomas.

Key Research Accomplishments

- Construction of the human wild type Stat5a expression vector
- Construction of the human constitutively active Stat5a expression vector